MAPK pathway and B cells overactivation in multiple sclerosis revealed by phosphoproteomics and genomic analysis

Kotelnikova, Ekaterina ; Kiani, Narsis A ; Messinis, Dimitris ; Pertsovskaya, Inna ; Pliaka, Vicky ; Bernardo-Faura, Marti ; Rinas, Melanie ; Vila, Gemma ; Zubizarreta, Irati ; Pulido-Valdeolivas, Irene ; Sakellaropoulos, Theodore ; Faigle, Wolfgang ; Silberberg, Gilad ; Masso, Mar ; Stridh, Pernilla ; Behrens, Janina ; Olsson, Tomas ; Martin, Roland ; Paul, Friedemann ; Alexopoulos, Leonidas G ; Saez-Rodriguez, Julio ; Tegner, Jesper ; Villoslada, Pablo

Abstract: Dysregulation of signaling pathways in multiple sclerosis (MS) can be analyzed by phosphoproteomics in peripheral blood mononuclear cells (PBMCs). We performed in vitro kinetic assays on PBMCs in 195 MS patients and 60 matched controls and quantified the phosphorylation of 17 kinases using xMAP assays. Phosphoprotein levels were tested for association with genetic susceptibility by typing 112 single-nucleotide polymorphisms (SNPs) associated with MS susceptibility. We found increased phosphorylation of MP2K1 in MS patients relative to the controls. Moreover, we identified one SNP located in the PHDGH gene and another on IRF8 gene that were associated with MP2K1 phosphorylation levels, providing a first clue on how this MS risk gene may act. The analyses in patients treated with disease-modifying drugs identified the phosphorylation of each receptor's downstream kinases. Finally, using flow cytometry, we detected in MS patients increased STAT1, STAT3, TF65, and HSPB1 phosphorylation in CD19<jats:sup>+</jats:sup> cells. These findings indicate the activation of cell survival and proliferation (MAPK), and proinflammatory (STAT) pathways in the immune cells of MS patients, primarily in B cells. The changes in the activation of these kinases suggest that these pathways may represent therapeutic targets for modulation by kinase inhibitors.

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Author: Ekaterina Kotelnikova, PhD[1,2,3], Narsis A Kiani, PhD[4], Dimitris Messinis, PhD[5], Inna Pertsovskaya, PhD[1], Vicky Pliaka, BSc[5,6], Marti Bernardo-Faura, PhD[8], Melanie Rinas, PhD[7], Gemma Vila, BSc[1], Irati Zubizarreta, MD[1], Irene Pulido-Valdeolivas, MD[1], Theodore Sakellaropoulos, PhD[6], Wolfgang Faigle, PhD[9], Gilad Silberberg, PhD[4], Mar Masso, BSc[10], Pernilla Stridh, PhD[11], Janina Behrens, MD[12], Tomas Olsson, MD[11], Roland Martin, MD[9], Friedemann Paul, MD[12,13], Leonidas G Alexopoulos, PhD[5,6], Julio Saez-Rodriguez, PhD[8,9,12], Jesper Tegner, PhD[4,10,11], Pablo Villoslada, MD[1*]

[1]Institut d'Investigacions Biomèdiques August Pi Sunyer (IDIBAPS), Barcelona, Spain; [2]Kharkevich Institute for Information Transmission Problems, Russian Academy of Sciences, Moscow, Russia; [3]Clarivate Analytics, Barcelona, Spain; [4]Unit of Computational Medicine, Department of Medicine, Solna, Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; [5]ProtatOnce, Athens, Greece; [6]National Technical University of Athens, Greece; [7]Joint Research Centre for Computational Biomedicine (JRC-COMBINE), RWTH-Aachen University Hospital, Aachen, Germany; [8]European Molecular Biology Laboratory, European Bioinformatics Institute, UK; [9]University of Zurich, Switzerland; [10]Bionure Farma SL, Barcelona, Spain; [11]Department of Neurology, Karolinska University Hospital, Sweden; [12]NeuroCure Clinical Research Center and Department of Neurology, Charité University Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany; [13]Experimental and Clinical Research Center, Max Delbrueck Center for Molecular Medicine, Berlin, Germany; [10] Biological and Environmental Sciences and Engineering Division, Computer, Electrical and Mathematical Sciences and Engineering Division, King Abdullah University of Science and Technology, Saudi Arabia; [11]Science for Life Laboratory, Solna, Sweden; [12] Institute of Computational Biomedicine, Heidelberg University, Faculty of Medicine, and Heidelberg University Hospitial, BioQuant, Heidelberg, Germany

*__Corresponding author__: Pablo Villoslada, Centre Cellex 3A, Casanova 145, Barcelona 08036, Spain. pvilloslada@clinic.ub.es

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Abstract

Dysregulation of signaling pathways in Multiple Sclerosis (MS) can be analyzed by phosphoproteomics in peripheral blood mononuclear cells (PBMCs). We performed in vitro kinetic assays on PBMCs in 195 MS patients and 60 matched controls and quantified the phosphorylation of 17 kinases using xMAP assays. Phosphoprotein levels were tested for association with genetic susceptibility by typing 112 single-nucleotide polymorphisms (SNPs) associated with MS susceptibility. We found increased phosphorylation of MP2K1 in MS patients relative to the controls. Moreover, we identified one SNP located in the PHDGH gene and another on IRF8 gene that were associated with MP2K1 phosphorylation levels, providing a first clue on how this MS risk gene may act. The analyses in patients treated with disease modifying drugs identified the phosphorylation of each receptor's downstream kinases. Finally, using flow cytometry, we detected in MS patients increased STAT1, STAT3, TF65 and HSPB1 phosphorylation in $CD19^{+}$ cells. These findings indicate the activation of cell survival and proliferation (MAPK), and pro-inflammatory (STAT) pathways in the immune cells of MS patients, primarily in B cells. The changes in the activation of these kinases suggest that these pathways may represent therapeutic targets for modulation by kinase inhibitors.

Keywords: multiple sclerosis, autoimmunity, immunomodulatory drugs, phosphoproteomics, genetic susceptibility, signaling pathways, kinases, B cells.

Significance statement

The alteration of cell communication in the immune system of patients with Multiple Sclerosis (MS) can be analyzed by phosphoproteomics in immune cells. We performed in vitro assays on immune cells from MS patients and controls and quantified the phosphorylation of several kinases. We found increased phosphorylation of several MAPK kinases in patients, which were modulated by several genetic markers associated with the disease. By using flow cytometry, we detected altered phosphorylation of several kinases in B cells. These findings indicate the activation of cell survival and proliferation (MAPK), and pro-inflammatory (STAT) pathways in the immune cells of MS patients, primarily in B cells. The changes in the activation of these kinases suggest that these pathways may represent therapeutic targets for modulation by kinase inhibitors.

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Introduction

Analyzing signaling pathways in patients with Multiple Sclerosis (MS) may provide insights into processes likely to drive the immune cell's response, as well as to influence the effects of drugs[1]. Phosphoproteomic analyses provide opportunities to evaluate the activation of signaling cascades, and such studies may help to identify the pathways activated in cells[2]. Mass spectrometry is the technique most commonly used to identify new phosphosites, whereas xMAP or flow cytometry are often used to evaluate the phosphorylation of larger numbers of kinases in vitro or ex vivo[3].

In MS, the immune system is chronically activated leading to specific damage of the CNS[4, 5]. GWAS studies suggest that the genetic susceptibility to suffer MS is mainly due to polymorphisms in genes associated with the immune system, consistent with an autoimmune pathogenesis[6]. In addition, also lifestyle/environmental factors are likely to act through effects on the adaptive arm of immunity, in view of their potent interactions with HLA class II and I genes, which are the restriction elements for CD4+ and CD8+ T cells, respectively[7]. Several pathways in the immune system have been implicated with MS pathogenesis, including those driven by the TCR, IL2, IL7, TNFa or NFkB [1, 8, 9], yet we still lack a comprehensive understanding of how immune pathways are truly involved in MS.

Immunomodulatory drugs significantly affect signaling in their target cells after receptor or target engagement. For this reason, changes in the phosphoprotein network may represent a sensitive read-out of drug activity and of the response of immune cells to the drug[3]. Generating better information as to how MS alters cell signaling will benefit the development of new therapies to combat this disease.

In this study we assessed the phosphorylation of key kinases and other proteins in signaling pathways associated with MS[1]. Phosphorylation was assessed by performing xMAP assays in vitro on PBMCs isolated from a crossectional cohort of MS patients and healthy controls (HCs) following perturbation with several stimuli and drugs. Moreover, we genotyped 112 single nucleotide polymorphisms (SNPs) associated with MS[6] in order to evaluate the influence of genetic background on

the phosphorylation in these pathways. Immune cell subtype was also characterized in a subgroup of patients by flow cytometry.

Results

Phosphoproteomic signatures in MS patients

In order to search for signaling pathways in the immune system that are differentially activated in patients with MS, we analyzed the phosphorylation of kinases in PBMCs from MS patients and sex and age-matched HCs. We studied a cross-sectional cohort of 195 MS patients and 60 HCs (see clinical characteristics in **Table S1**) recruited in the CombiMS project. We obtained phosphoproteomic information that conformed the quality control (QC) assessment from 169 individuals (132 MS patients and 37 HCs) and genotyping data from 154 out of those 169 individuals (122 MS patients and 32 HC; 14 subjects were excluded because genotyping did not pass QC checks). The final cohort was representative of the recruited cohort and between sites (see SI Appendix, **Table S1**).

The phosphoproteomic assays were designed to study signaling deregulation using dynamic network modeling. This linked study compiled and presented a manually curated immune- and MS-based signaling network[10]. After topological analysis, we screened the 70 antibodies for the xMAP assays that maximized coverage of that network, identifying 30 antibodies with good signal to noise ratio. We selected a set of 17 assays that were suitable for the multiplex assays. The 17 assays used in the vitro assays were: AKT1, CREB1, FAK1, GSK3A, HSPB1, IKBA, JUN, MK03, MK12, MP2K1, PTPN11, STAT5, STAT1, STAT3, STAT6, TF65 and WNK1 (**Figure 1** and **Table S2**). Considering that phosphorylation is a dynamic event that takes place soon after stimulation and that is sensitive to different stimuli, we performed ex vivo assays in PBMCs stimulated with 19 different stimuli known to activate such kinases or that are relevant for MS pathogenesis or therapy, including cytokines, metabolites and drugs (see **Figure 1** and **Table S3**). In order to capture the differences already present at baseline and those that require activation of specific pathways, phosphorylation was analyzed at baseline (unstimulated), and 5 and 25 min after stimulation (stimulated). These time points after stimulation were chosen as they showed the strongest phosphorylation (either 5 or 25 min), as

well as the stimulus that produced the most significant differences (**See SI Appendix, Figure S1**). We checked that no center or batch effect was present in the normalized phosphorylation levels by performing principal component analysis. In addition, we genotyped our cohort for 112 SNPs[6] associated with susceptibility for MS (**Table S4**) and these genotypes were used to evaluate the effect of such SNPs on phosphorylation levels (see SI Appendix file S1 for raw genotyping data).

First, we analyzed the baseline differences in phosphoproteins between MS patients and HCs. We found a significant increase in the baseline phosphorylation of MP2K1 in MS patients compared to controls after adjustment for multiple comparisons (FDR). MP2K1 is an element of the MAPK signaling pathway that promotes cell survival and inhibits apoptosis, in particular through the NFkB cascade. Indeed, this difference remained significant in the relapsing-remitting (RRMS) subgroup after adjusting for multiple comparisons (Wilcoxon test RRMS vs control p-value=0.0016, FDR=0.03; Wilcoxon test all MS patients vs control p-value=0.0034, FDR=0.065: **Figure 2**). MKO3, GSK3A, JUN and STAT3 phosphorylation levels at baseline were also different between MS and controls in the unadjusted analysis, although such differences were not significant after correction for multiple testing (**Table S5**). In addition, we found differences in kinase phosphorylation levels when the cells were subjected to distinct stimuli in vitro, although these effects were not significant after adjusting for multiple comparisons.

Then, we analyzed how the MS susceptibility genotype influenced phosphorylation levels (**Figure 3 and S2**). After FDR correction we found a significant influence of the TT genotype of the rs666930 SNP (located in the phosphoglycerate dehydrogenase gene -PHGDH) on baseline MKO3 and MAP2K1 phosphorylation in MS patients. Similarly, the CT genotype of the rs35929052 SNP (located on Interferon regulatory factor 8 -IRF8 gene, a key target of vitamin D receptor[11, 12]) had a significant influence on MAP2K1 phosphorylation after stimulation with vitamin D3 (**Table 1**). We found significant effects of several SNPs on the phosphorylation differences between MS and HCs (ANOVA adjusted p-value<0.05). 12 kinases were shown to be involved in these effects, including AKT1, FAK1,

GSK3A, JUN, MKO3, MP2K1, STAT1, STAT3, STAT5, STAT6, TF65, WNK1 (**Table S6**), although only those ones described above for MKO3 and MP2K1 (Table 1) remained significant for the allele-specific pairwise comparisons.

Indeed, we assessed whether disease subtype was associated with differential phosphorylation, comparing RRMS and progressive MS with HCs. We found the same differential phosphorylation at baseline for MAP2K1 in RRMS patients relative to HCs but not in PMS (**Figure 2**). Regarding the analysis of phosphorylation levels and its association with MS associated SNPs for disease subtypes, we found significant differences in JUN phosphorylation after insulin stimulation in RRMS individuals with the GG genotype for the rs11554159 SNP, located in the Interferon gamma-inducible protein 30 (IFI-30) gene. Similarly, the effect of the CT genotype of the rs35929052 SNP on the phosphorylation MP2K1 following VitD3 stimulation was significantly different between RRMS patients and HCs (**Table 1**).

Finally, we assessed the biological relationship of the found genetic and phosphoprotein associations by performing a protein network analysis (see SI Appendix methods). This pathway analysis indicates that the potential influence of the SNP rs35929052 located in the IRF8 gene on MAP2K1 kinase activity could be mediated by the interaction of IRF-8 with TRAF6 and with changes in the transcription of EGR1 and PPAR-gamma. Moreover, the analysis also indicates that the influence of SNP rs666930 located in the PHGDH gene on MAPK3 can be explained by a physical interaction between PHGDH protein with both EGFR and MAP3K3 proteins (**Figure 4**).

Phosphoproteomic signatures associated with immunomodulatory therapies

In order to assess the effects of DMDs approved for the treatment of MS on the signaling network in immune cells from patients with MS, we analyzed the phosphoproteomic profile of the 17 kinases in patients treated for more than one year with a given DMD and compared it to that in untreated RRMS patients (see **Table S1** for clinical details). We performed the analysis for the most common disease modifying drugs (DMDs) at the time of the analysis, namely fingolimod (FTY), natalizumab (NTZ), interferon beta1a s.q. 44 mcg (IFNB1a) and glatiramer

acetate (GA). Teriflunomide and dimethyl-fumarate were not included in the analysis because there were insufficient patient numbers treated with these DMDs at our centers by the timing of recruitment.

Regarding the changes in phosphorylation induced by FTY, the phosphorylation of STAT1, MKO3 and PTPN11 after stimulation differed in FTY-treated patients (n=13) compared to untreated RRMS patients (n=58: **Table S7**). Moreover, we found significant differences between FTY-treated and untreated RRMS patients after adjustment for genotype, specifically in terms of IKBA phosphorylation for the AG rs11581062 SNP, TF65 phosphorylation for the GG rs2293152 SNP, and CREB1 phosphorylation for the CT rs6498184 SNP (**Table S7B and Figure 3B**). In summary, in FTY-treated patients we identified several kinases downstream of the S1P receptor that are implicated in the MAPK or NFKB pathway (e.g. MKO3, IKBA, TF65) as well as several kinases that do not participate in the S1P receptor pathway, yet that were related to pathways involved in the immune response (e.g. STAT1, STAT3, STAT5, STAT6).

Patients treated with NTZ (n=19) showed differential phosphorylation of STAT3, STAT5, STAT6 and MP2K1 compared to untreated RRMS patients (n=58: **Table S8)**. After adjusting for the genotype, we found an association with JUN, AKT1, FAK1, GSK3A, HSPB1, PTN11, STAT1 phosphorylation and NTZ therapy (**Figure 3B**). The VLA4 (ITGA4) receptor interacts with the Ras/MAPK, PI3K and NFKB pathways, and our results implicate the activation of several members of the Ras/MAPK (MP2K1, HSPB1) as well as other kinases associated with overall immune activation (STAT3, STAT5, STAT6).

Patients treated with IFNB1a (n=23) showed distinct STAT1 phosphorylation compared to untreated RRMS patients (n=58), with a dependence on the AC rs759648 SNP (adjusted p-value=0.0088). After adjusting for genetic susceptibility, we observed an association between IFNB therapy and the phosphorylation of JUN, AKT1, FAK1, GSK3A, MKO3, MP2K1, STAT1, STAT3, STAT5 and TF65 (**Figure 3B**). Considering that JAK/STAT participate in type 1 interferon receptor pathways, our findings suggest that several kinases in this pathway are

likely to be activated (STAT1, STAT3, STAT5), as well as those in other pathways like PI3K, MAPK or NFKB, supporting the pleiotropic immunomodulatory activity of IFNB.

Finally, the phosphorylation of STAT6 in response to insulin differed in patients treated with GA (n=10) compared to untreated RRMS patients (n=58: pSTAT6 levels in GA treated patients 0.206585; untreated patients 0.011394; adjusted p value= 0.009). The JUN, CREB1, FAK1, GSK3A, STAT1, STAT5 phosphoproteins were more evident in GA-treated patients after adjustment for SNPs of MS susceptibility (**Figure 3B**). Although GA seems to primarily mediate the induction of GA-specific regulatory T cells, it also appears to display broad immunomodulatory effects[13]. Thus, the activation of such kinases may be related with the immunomodulatory effects of such cells.

Analysis of kinase phosphorylation in immune cell subtypes by flow cytometry

In order to analyze the cell subtypes responsible for the differences in phosphorylation, we analyzed the phosphoproteins levels in PBMCs by flow cytometry. Phosphorylation was assessed in a representative subgroup of 47 MS patients and 22 HCs from the original cohort (**Table S1**). We analyzed the phosphorylation of 7 out of the 17 kinases used for the xMAP assays for which cytometry assays passed QC and showed a good signal to noise ratio: CREB1, HSPB1, IKBA, MK03, MK12, STAT1, STAT3, STAT5, TF65 and WNK1 (**Table S9** shows the list of antibodies used and **Table S10** show the list of ex vivo assays conducted on each disease subgroup). However, MP2K1 was not studied in this cytometry substudy due to the lack of antibodies that passed the QC checks and provide good signal to noise ratio. We found significant differences in the phosphorylation of HSPB1 in monocytes ($CD33^+$ cells) and STAT3 in B cells ($CD19^+$ cells) from MS patients relative to HCs. Moreover, there were significant differences in the phosphorylation of STAT1, STAT3 and TF65 in $CD19^+$ cells in PPMS patients compared to the HCs. In terms of the differential phosphorylation related to the use of DMDs, we found significant differential expression of HSPB1 in $CD19^+$ cells in patients treated with FTY (**Table S11**).

Discussion

We have performed a comprehensive analysis of the phosphoproteomic changes in immune cells from patients with MS. We found distinct patterns of kinase phosphorylation in patients with MS, mainly involving the MAPK pathway but also affecting the NFkB and STAT pathways. Such pathways are known to be critical for cell survival and proliferation, cell adhesion and chemotaxis, and the pro-inflammatory response of the immune cells. The phosphoproteomic data was generated to perform logic network modeling[10]. With this approach, pro-inflammatory and pro-survival pathways were found to be deregulated. Further, these pathways were used for combination therapy prediction and subsequently validated. Hence, both studies jointly demonstrate that such differential activation can potentially benefit from new immunomodulatory therapies for MS and other autoimmune diseases using approved and novel kinase inhibitors.

Our studies highlight the prominent role of the MAPK pathway in the peripheral immune system of patients with MS, primarily the ERK sub-pathway. This importance of the MAPK pathway is illustrated not only by the increased phosphorylation of MP2K1 and its downstream target MK03 but also, by that of the p38 sub-pathway, as reflected by HSPB1 phosphorylation. MP2K1 (also known as CFC3, MEK1, MKK1, MAPKK1 or PRKMK1) promotes cell survival and inhibits apoptosis, in particular through the NFkB cascade[14]. MAP2K1 is activated through KRAS and BRAF activity (e.g. after EGFR activation), and it phosphorylates ERK kinases and interacts with the C-Raf, phosphatidylethanolamine binding protein 1, MAP2K1IP1, GRB10, MAPK3, MAPK8IP3, MAPK1MP1 and MAP3K1. MK03 (also known as ERK1, MAPK3 or PRKM3) is phosphorylated by MAP2K1, contributing to the pro-survival signaling in this pathway. MK03 is important to induce T cell anergy and it acts as a negative regulator of dendritic cells, controlling their capacity to prime T cells towards an inflammatory phenotype[15]. HSPB1 (Hsp27) is a small heat shock protein that displays chaperone activity, inhibits apoptosis, and regulates cell development and differentiation[16]. HSPB1 is also part of the MAPK pathway, as it is activated by the p38 kinase MK2-3 (although it can also be activated by MK5, PRAK, PKCγ and PKD) and plays an important role in inflammation[16]. Moreover, HSPB1 is overexpressed by astrocytes in MS plaques[17],

probably in response to the inflammatory stress that helps protect the CNS and prevent apoptosis[18]. Due to the key involvement of the MAPK pathway in cancer (RAS, BRAF, CRAF, MEK1 or MEK2 mutations), MEK1 inhibitors like trametinib or cobimetinib have been approved for the treatment of BRAF-mutated melanoma and new ERK1 inhibitors are being actively sought[19]. Therefore, there is an opportunity to test approved MEK1 inhibitors, or new ones under development, for their capacity to modulate the immune response in MS and other autoimmune diseases[14].

MS genetic susceptibility also seems to be associated with signal pathway activation as we found several SNPs associated with altered kinase phosphorylation in immune cells. At present we lack sufficient understanding of how genetic polymorphisms regulate protein phosphorylation in a direct or indirect manner, although it is well known that mutations in specific kinases alter their activity and the phosphorylation of their downstream targets[20]. Convincing data has appeared with regard to the TYK2 gene polymorphism where a protective variant reduces cytokine signaling, without compromising the defense against infections[21]. Up to 10% of the human genome encodes for proteins that modulate phosphorylation or other types of post-translational protein modifications. Mutations in ligands, receptors and adaptors affect protein phosphorylation indirectly, thereby suppressing or enhancing the activation of signaling networks[20]. Indeed, recent GWAS studies have shown association signals between SNPs at loci linked to genes encoding kinases and related proteins, and numerous complex and common disease phenotypes[22], including the association of SNPs of TyK2, RPS6KB1, MAPK1, MAPK3, RELA, NFKB1, STAT3 or STAT4 with MS[6, 8, 21, 23-26]. These polymorphisms may be implicated in the direct regulation of signaling pathways or they may serve as markers of regulatory elements that segregate with MS. We here provide preliminary evidences suggesting that MS risk gene SNPs may be associated functionally to kinase signaling.

The selection of SNPs used in this study was based on the GWAS studies of 2013[6], but more recent available studies have increased the number of SNPs. In addition, fine-mapping the SNPs associated with phosphoprotein levels would help to characterize the biological basis of the statistical association identified here. For

this reason, new studies with high genetic coverage would be required to further characterize the influence of genetic susceptibility in the signaling pathways involved in MS pathogenesis[9].

The effects recorded preferentially on B cells are of great interest, since the depletion of CD20+ cells has demonstrated unexpected high efficacy in MS[27]. Several mechanisms for the effect of B cells have been presented, like the B cells capacity to present antigens, their cytokine production, and development into antibody producing cells[28]. We here suggest one more potential piece of evidence in the puzzle; a preferential activation of kinases in B cells.

We identified altered phosphorylation of several kinases downstream of the S1P receptor that are involved in the MAPK or NFKB pathways in patients treated with FTY. Similarly, we found alterations in the phosphorylation of proteins in the VLA4 receptor pathways in patients treated with NTZ, such as Ras/MAPK, or the activation of several STATs that participate in type I interferon pathways. Nevertheless, we also observed altered phosphorylation in other pathways. Considering the main mechanism of action of some drugs (FTY and NTZ) is preventing cell migration, it remain unclear to which extent our findings are a direct consequence of the activation of drug targeted pathways or a reflection of the immune system adaptation to changes in immune cells dynamics. However, the crossectional design of our study and the influence of such drugs in PBMCs composition prevents to establish a definite causality between drug use and kinase activation, and for this reason prospective validation studies are required to confirm such findings. Indeed, longitudinal studies are also required for defining the role of these kinases in the response to therapy. Anyway, phosphoproteomics is evolving as the method of choice in some areas of the drug discovery process, and it is has also become more suitable for the discovery of novel targets or biomarkers[3].

This study has several limitations, such as the use of a medium through-put approach such as xMAP, or flow cytometry with limited number of assays available with a good signal to noise ratio. Indeed, we have measured only levels of phosphorylated proteins, without solving whether this was due to increased phosphorylation or by higher abundance of the kinase or both. However, the

kinases we have included in the analysis are known to participate in pathways previously associated with MS and autoimmunity, supporting the biological relevance of our results[1]. The selection of the stimuli was based in the literature pointing for T cell activation and for this reason specific activators of B cell response were not included. Our analysis simplified signaling process to the analysis of phosphorylation events but not other post-translational changes, employing few time-points and conditions. Moreover, our study was cross-sectional, whereas, longitudinal analysis will be required in order to define the response to each of the DMDs at the clinical level and being used for identifying the role of several pathways and kinases in the response to therapy. In addition, we performed many statistical tests and obtained results adjusted for statistical multi-testing across the entire study, although this approach does not allow us to exclude false positive results. Indeed, the use of multiple testing corrections decreases the power of the study for detecting true associations and, for example, only MP2K1 remained significant in the comparison between patients and controls. However, despite such limitations, our study provides evidence of the specific activation of the MAPK pathway in the immune cells of MS patients, which may promote the development of MAPK targeted therapies for MS.

Methods

See SI Appendix, Supplementary Text for extended method explanations.

Subjects

We recruited 255 subjects, 195 patients with MS and 60 healthy controls matched for age and sex with the RRMS group, from 4 MS centers (**Table S1**): Hospital Clinic of Barcelona (n=69); Karolinska Institute (n=64); University of Zurich (n=40) and Charité University (n=82). Patients fulfilled the McDonald 2005 criteria[29] and their disease subtype was defined using Lublin criteria[30]. Patients were allowed to receive any therapy and in the previous 6 months they had not required any adjustment to their therapy.

xMAP assays

xMAP assays were performed blinded at ProtAtOnce (Athens, Greece). We optimized the assays from a list of 70 candidates based in QC checks and the signal to noise analysis and obtained a final list of 17 phosphoproteins with optimized assays (**Table S2**). We used a set of 19 stimuli that included pro-inflammatory and pro-oxidant stimuli; immunomodulatory stimuli; neuroprotectants and anti-oxidants; and DMDs (**Table S3**). Such stimuli are known to activate several pathways known being associated with MS pathogenesis (e.g. MAPK, NfKB or STAT) or trigger the activation of DMDs receptors (**Fig. 1**). The samples were collected at the baseline (time 0), and 5 and 25 min after stimulation. All the data was normalized after reading the signals. Changes in phosphorylation for each protein and each patient were calculated with respect to the control conditions[31]. The phosphorylation of each protein in response to stimulation was defined as the log2 of the response to the stimulus relative to the response to the medium.

Cytometry

Patient's samples were washed three times and stained with the antibodies (**Table S6**). Four subtypes of immune cells were identified and gated: $CD4^+$ cells; $CD8^+$ cells; B cells (CD19); and monocytes (CD33).

Genotyping

Genotyping was performed on DNA samples collected from the subjects, assessing SNPs previously validated as associated with MS[6]. The final list includes 112 SNPs, including 1 SNP associated with HLA-DRB1*1501 (**Table S4**).

Statistical and bioinformatic Analysis

We compared pairs of groups using a Wilcoxon test (for baseline) or a T-test (for responses to stimuli) using R software. Genotype to phosphoprotein levels associations were tested using two-way ANOVA, and with each SNP as a first independent factor and the patient group as a second factor. The false discovery rate was always corrected using the Benjamini-Hochberg procedure (reported as significant when FDR <0.05). Protein network analysis is described in SI Appendix, Supplementary Text.

Ethics statement

This investigation has been conducted according to Declaration of Helsinki principles. The study was approved by the Ethics Committee of the Hospital Clinic of Barcelona, Hospital San Martino of Genova, Charité University of Berlin and University of Oslo. Patients were recruited by neurologists after they had provided their signed informed consent prior to inclusion in the study.

***Data and materials availability*:** The phosphoproteomic dataset can be found at https://github.com/saezlab/combiMS, together with the code allowing network modeling of signaling pathways[10].

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Author's contribution: IZ, ISP, WF, PS, TO, RM, FP and PV recruited patients and collected clinical information; JSR, LA, PV, TO, RM, MM and FP designed and supervised the research; DM, VP, TS and LA developed the xMAP assays; MBF, TS, LGA and JSR processed and normalized the phosphoproteomic data; MR curated the phosphoproteomic dataset; GV, IP, IZ, GS, PS, JB and WF performed the *in vitro* assays; EK, NAK, JT, and PV analyzed the data; EK and PV prepared the manuscript, which was revised by TO, MR, FP, and MM; and the statistical analysis was performed by EK.

Competing interest: DM is an employee of ProtATonce; TO received honoraria for lectures and/or participation on advisory boards, as well as unrestricted multiple sclerosis research grants from Allmiral, Astrazeneca, Biogen, Genzyme, Merck and Novartis; RM received grants and personal fees from Biogen Idec, personal fees from Genzyme Sanofi Aventis, grants and personal fees from Novartis, personal fees from Merck Serono, Bionamics, all for work unrelated to that submitted; RM received honoraria for lectures and/or participation on advisory boards, as well as unrestricted multiple sclerosis research grants from Biogen, Genzyme, Merck, Celgene, Roche,

Novartis, Neuway and CellProtect, all for work unrelated to that submitted; FP received research grants and personal compensation from Alexion, Bayer, Chugai, Novartis, Merck, Teva, Sanofi, Genzyme, Biogen, and MedImmune; LGA is the founder and shareholder at ProtATonce; PV hold stocks and have received consultancy fees from in Bionure Farma SL, QMenta Inc, Health Engineering SL, Spiral Therapeutics Inc and Spire Bioventures Inc. All other authors have no competing interests to disclose.

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Figure Legends

Figure 1. The immune signaling network in MS. The figure shows the stimuli used for the in vitro assays (purple hexagons on the left), targeting receptors on the cell membrane (orange circles), although some stimuli directly target kinases or pathways (e.g. H_2O_2 directly induces oxidative stress). Membrane receptors are linked to intracellular kinases (light brown circles) as part of their intracellular signaling networks. The specific phosphoproteins tested in these assays are shown as red circles associated to the master kinase. Finally, kinases influence cellular and molecular processes. Stimulation (e.g. phosphorylation) is indicated by green arrows whereas inhibitory interactions (e.g. dephosphorylation) is indicated by a red T link.

Figure 2. MP2K1 phosphorylation in PBMCs from MS patients and controls. Phosphorylation of MP2K1 in PBMCs from MS patients and controls, as assessed by xMAP and compared with a Wilcoxon test: * $p<0.05$; ** adjusted $p<0.05$.

Figure 3. The phosphoproteomic signature in MS. The lines in the circle graph show the pairs of stimuli/phosphoproteins found to be significant and the corresponding MS susceptibility genes (ANOVA test for association between kinase levels and SNPs, Benjamini correction for multiple testing). The outer circle shows the 17 phosphoproteins analyzed and the 19 stimuli used, while the inner circle shows the 112 SNPs tested (color coded for the mean allelic distribution) where the SNP indicated by the link is referred to by its letter code. a) Phosphoproteomic signature in MS, healthy controls, and the RMS and PMS subtypes of MS. b) Phosphoproteomic signature of the DMDs compared to untreated RRMS patients.

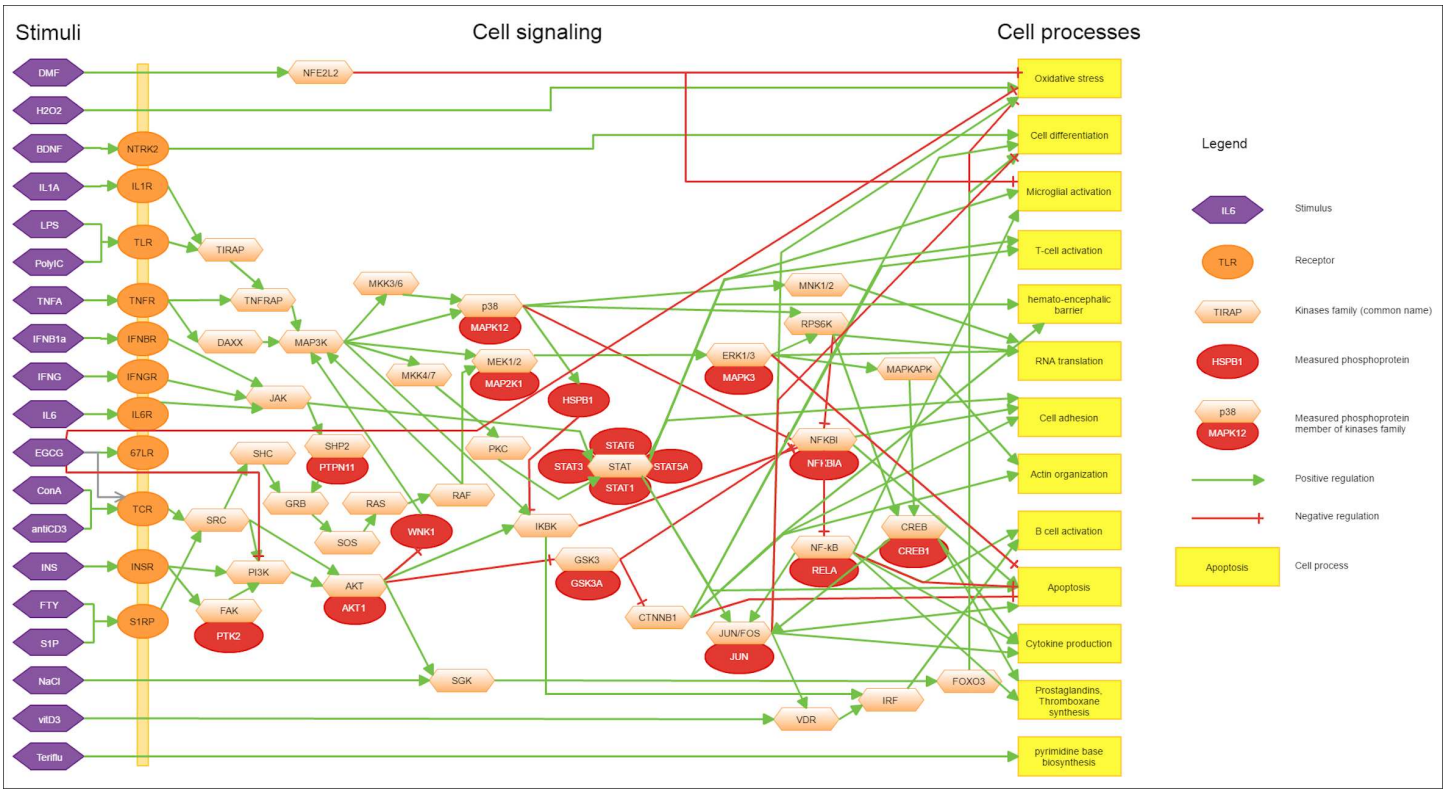
Figure 4. Protein network analysis of phosphoproteins and SNPs associated with MS. The graph shows the proteins from either genes containing MS-susceptibility SNPs (in red) or phosphokinases (in blue) found associated in our analysis. a) first-order physically interactions of the studied proteins identified on the iRefIndex and MetaBase/MetaCore database. b) transcriptional regulation (dashed lines) and physical interactions (solid lines) of the associated proteins

identified using the TieDie algorithm on the background of directed MetaBase network.

Table 1. Differential phosphorylation in PBMCs from MS patients relative to healthy controls. The table shows the kinases that were more strongly phosphorylated in MS patients relative to the HCs, indicating the stimulus used in the in vitro assays and the susceptibility SNPs (Benjamin correction for multiple tests). The results are shown as the mean ± SD. Unstimulated basal phosphoprotein levels were compared using a Wilcoxon test, whereas a phosphorylation after stimulation was normalized and compared using a T-test.

	Stimulus	**SNP: allele**	**phosphorylation levels**		**p**	**Adjusted p**
MS			**MS**	**HCs**		
MKO3	baseline	rs666930: TT	937 ± 480	463 ± 86	0.0011	0.004
MP2K1	vitD3	rs35929052: CT	0.44 ± 0.42	-0.24 ± 0.2	1.32E-05	0.0016
	baseline	rs666930: TT	3849 ± 1794	2041 ± 646	0.0019	0.04
RRMS						
JUN	INS	rs11554159: GG	0.12 ± 0.14	-0.017 ± 0.1	0.00022	0.027
MP2K1	vitD3	rs35929052: CT	0.48 ± 0.47	-0.24 ± 0.2	6.71E-05	0.008

VitD3, vitamin D3; INS, insulin



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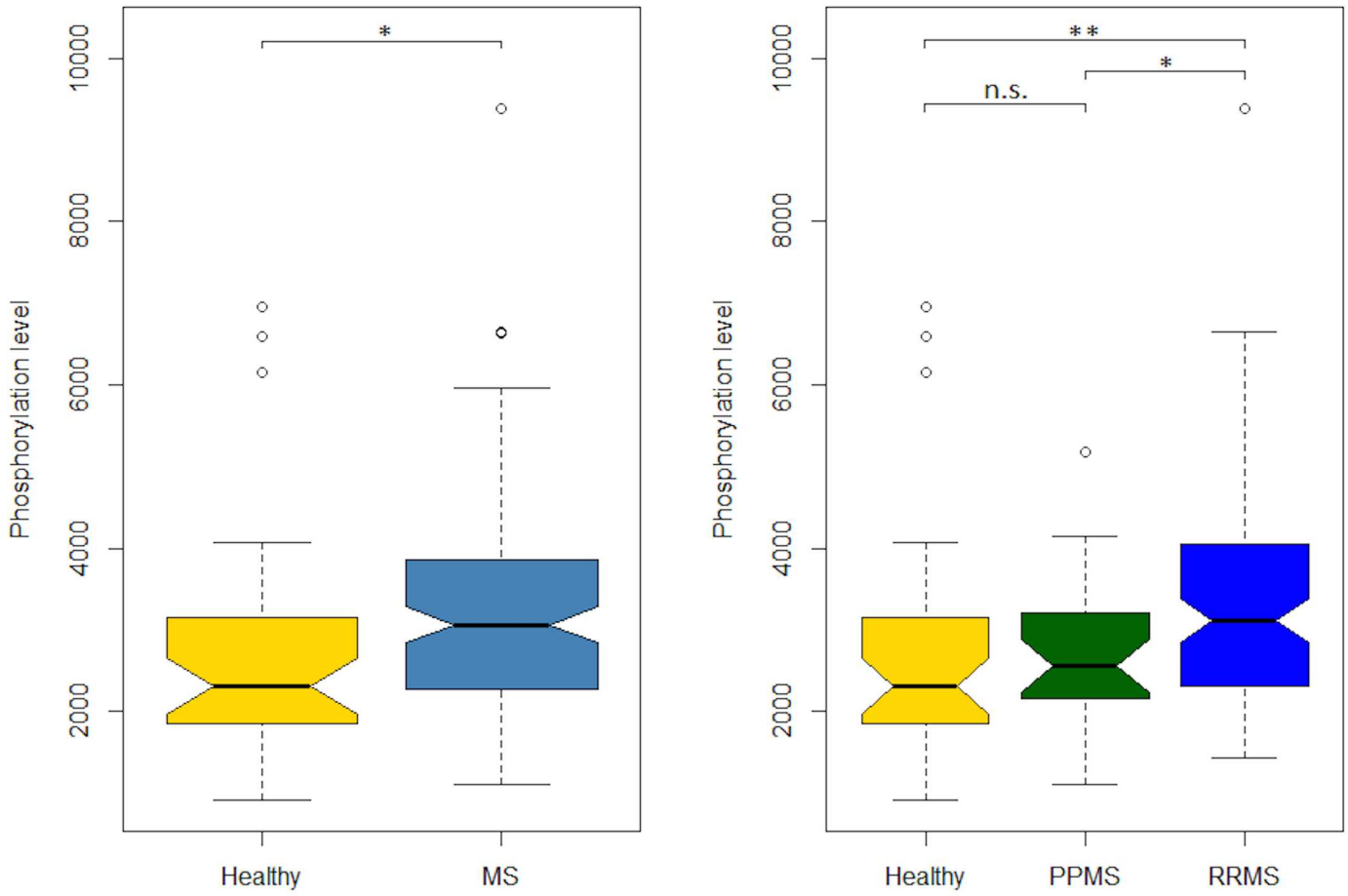

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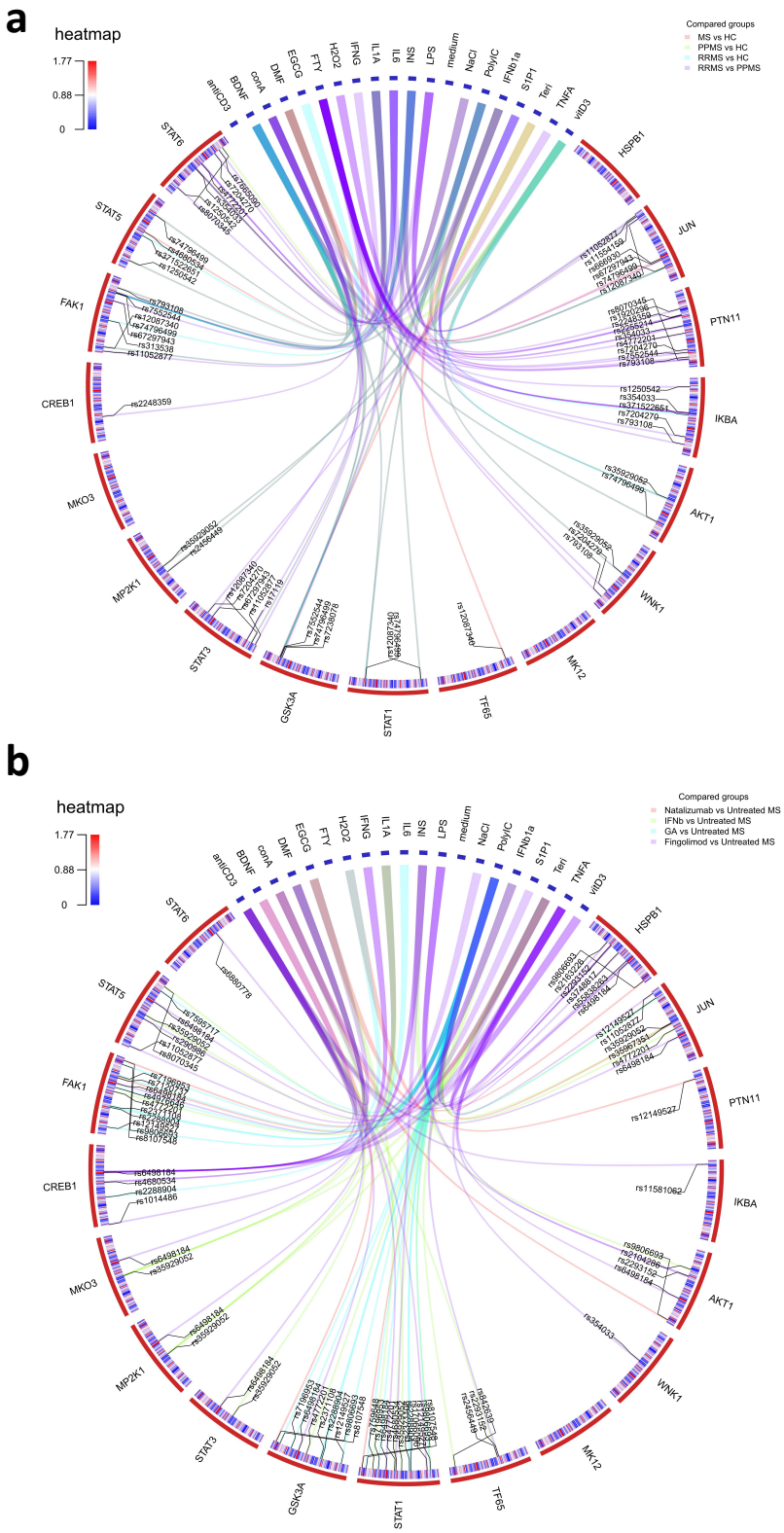

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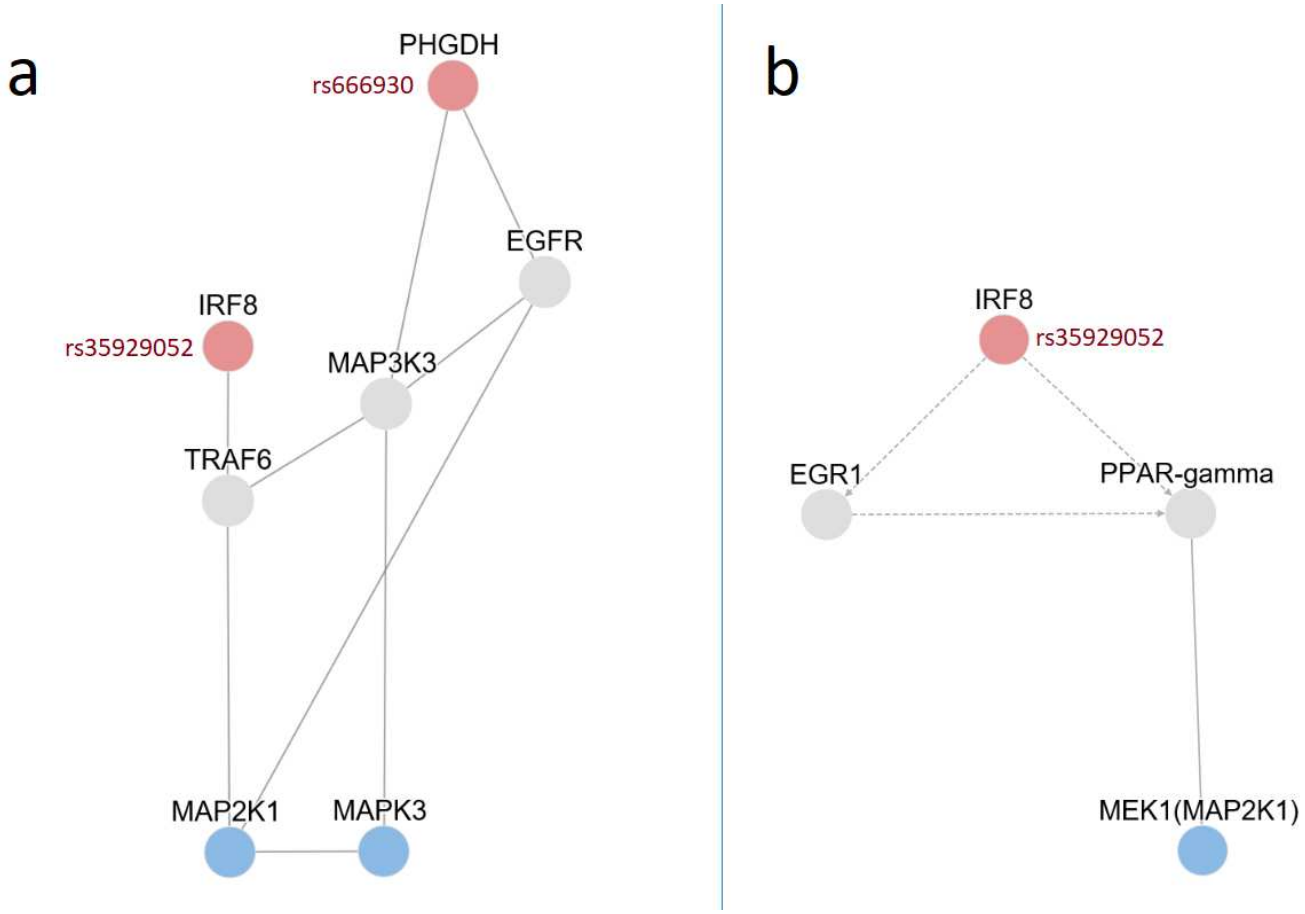

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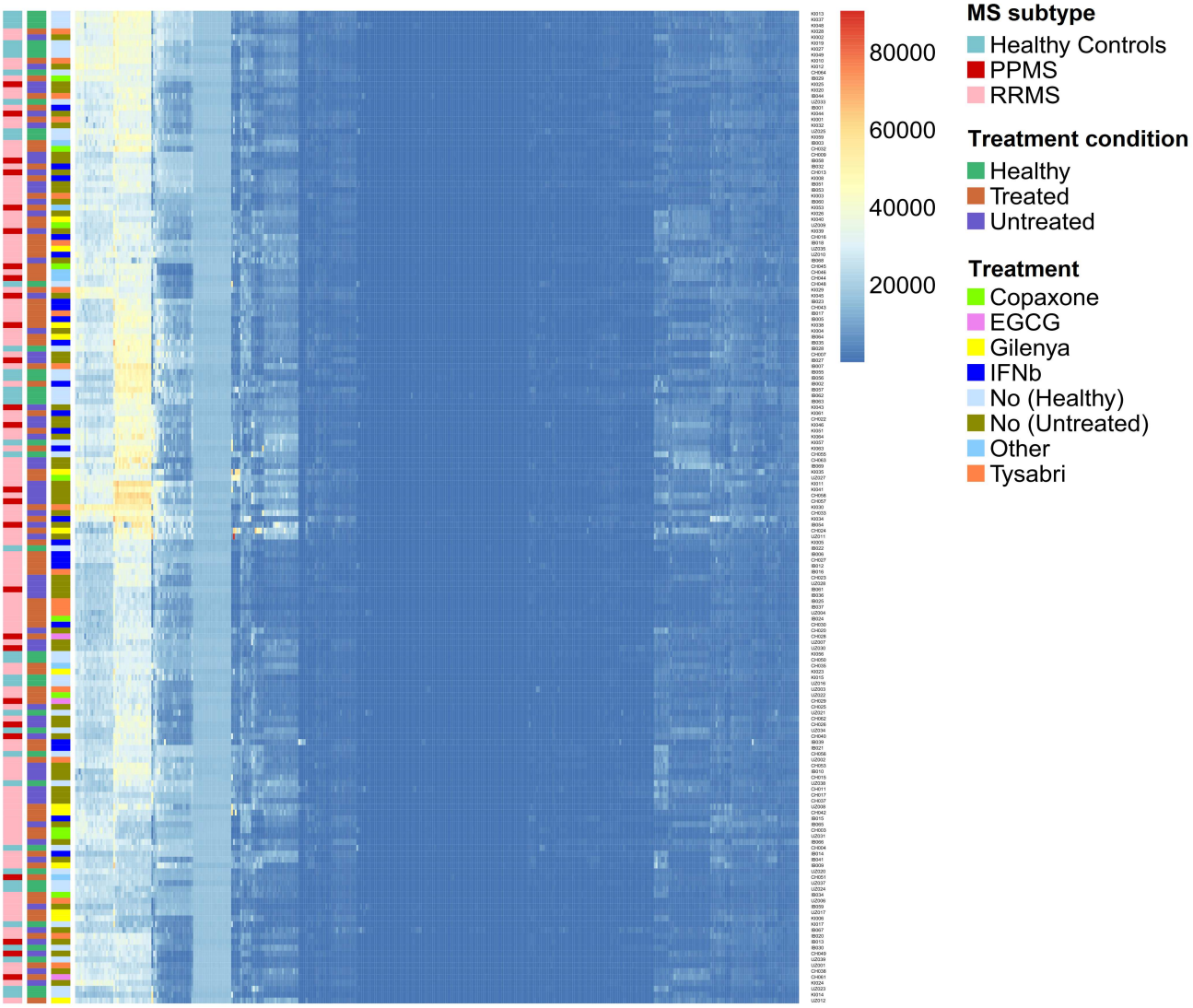
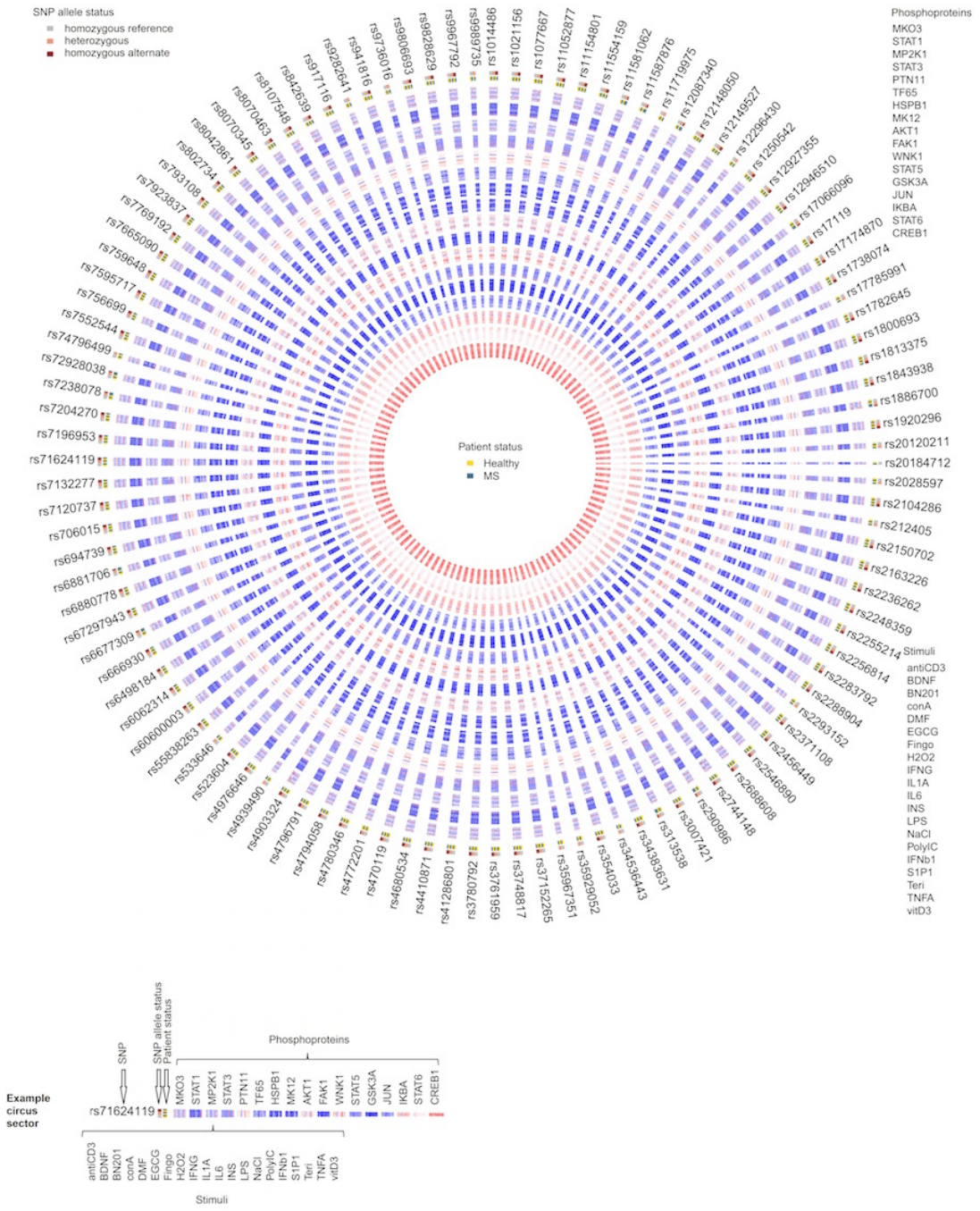


Supplementary figure S1. Phosphoprotein levels in healthy controls and in patients with MS. Heat map of the raw phosphorylation levels (after normalization) annotated for different disease subtypes and treatments. Imputation of the missing values using knn: k=10 for each cpp 10 nearest patients/samples.



Supplementary figure S2. Allelic distribution of MS susceptibility SNPs in MS patients and controls, and their association with the phosphoprotein levels. For each SNP we show 6 sub-sectors: 3 for each allele variant for the two groups (MS and HC). The heat map shows the level of one of the 17 phosphoproteins in response to the 19 different stimuli for each radius. Each sector related to a given SNP at a specific radius could be considered as a separate heat map of the phosphorylation response of the selected kinase stratified by genetic variance at the position of this SNP. Circular tracks from out to in: 1) SNP name; 2) allele status of the SNP: reference homozygous (grey), heterozygous (orange), alternative homozygous (red); 3) disease status, MS (blue) or HC (yellow); 4) the combination of allele status and disease status defines the number of rows in the heat map for each specific phosphoprotein; 5) heat maps of the response of the specific phosphoproteins as a function of the overall variance in the data (from out to in): MKO3, STAT1, MP2K1, STAT3, PTN11, TF65, HSPB1, MK12, AKT1, FAK1, WNK1, STAT5, GSK3A, JUN, IKBA, STAT6, CREB1. Each phosphoprotein heat map has 4-6 rows (number of allele statuses * number of disease statuses) and 20 columns, related to the stimuli: anti-CD3, BDNF, ConA, DMF, EGCG, FTY, H_2O_2, IFNG, IL1A, IL6, INS, LPS, NaCl, PolyIC, IFNb1a, S1P1, Teriflunomide, TNFA, vitD3. The color represents the phosphorylation response (log2 [phosphorylation of the selected kinase after stimulation/ phosphorylation of the selected kinase in the medium]). Heat map: blue - lower values, red - higher values averaged over the cohort with the specific allele/disease status.